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Interaction of Alamethicin with Lecithin Bilayers: A ^{31}P and ^{2}H NMR Study†

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ABSTRACT: The interaction of alamethicin with artificial lecithin multilamellar dispersions was investigated by nuclear magnetic resonance (NMR) and Raman spectroscopies. ^{31}P NMR studies revealed perturbation of the lipid head groups in the presence of the icosapeptide. Simulation of the ^{31}P NMR spectra indicated that the observed spectral changes could be attributed to slight variations in the average tilt angle of the head groups. In contrast, no noticeable effect of the peptide on the segmental order of the hydrophobic acyl chains of the lipid molecules was detected by ^{2}H NMR and Raman spectroscopic measurements. Taken together, these results indicated that, in the absence of a transmembrane electric potential, alamethicin interacts primarily at the water–lipid interface without significant insertion or incorporation into the bilayer leaflet.

Alamethicin is an antibiotic icosapeptide extracted from the fungus *Trichoderma viride*, which exhibits voltage-gated ionic conductance in black lipid membranes (Latorre & Alvarez, 1981). On account of its structural simplicity, this molecule can serve as an ideal model system to study the mechanism of gated ionic conductance through lipid bilayers. In a recent paper, we reported the structure of alamethicin (Banerjee et al., 1983) in methanol and aqueous methanol mixtures. On the basis of nuclear magnetic resonance (NMR)[1] measurements at 11.74 T, the peptide was shown to exist as a dimer in methanol, stabilized by intermolecular hydrogen bonds at the C-terminus. Dimerization in methanol was confirmed by proton spin–spin relaxation measurements (Banerjee & Chan, 1983). Proton amide coupling constants ($J_{NH-\alpha CH}$) indicate that the N-terminal end of the molecule is α-helical over nine residues, while the structure at the C-terminus corresponds to the β-pleated sheet. An important feature of this dimer is that one face is completely hydrophobic, while the other is lined with polar groups. Studies in water–methanol mixtures revealed that the secondary structure of the peptide remains unchanged in the more polar solvents. Extensive aggregation of the peptide was noted in water, however, and it was proposed that the dimer associates further to yield highly amphipathic micellar structures possibly via sequestering of the hydrophobic domains of the peptide away from water.

In the present paper, we describe a series of studies designed to elucidate the interaction of alamethicin with phospholipid membranes. There has been much debate over the years about the partitioning of the peptide into the bilayer membrane. Early studies have suggested that alamethicin is primarily a surface-active molecule with minimal partitioning into the hydrophobic core of the membrane in the absence of an electric field (Lau & Chan, 1975, 1976). Single channels can then be formed by the insertion of alamethicin into the bilayer upon the application of an electric potential. Others have proposed complete partitioning of alamethicin into bilayer membranes (Fringeli & Fringeli, 1979; Latorre et al., 1981). In fact, models for alamethicin conductance have been proposed which require preaggregation in the membrane (Boheim et al., 1983).

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[1] Abbreviations: NMR, nuclear magnetic resonance; DML, dimyristoylphosphatidylcholine; DML-d_{54}, bis(perdeuteriomyristoyl)phosphatidylcholine; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane.

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In an attempt to resolve this controversy, we have studied the interaction of alamethicin with multilayer membranes using a number of NMR probes that are sensitive to various parts of the bilayer membrane. ^{31}P NMR of the phosphate moiety of the lipid head groups can, in principle, give information on perturbations induced by the peptide near the water–bilayer interface; ^{2}H NMR, in turn, can be used to monitor the perturbations of the lipid acyl side chains by alamethicin. We have augmented these NMR results with parallel Raman measurements, another spectroscopic technique capable of providing information on the fluidity of acyl chains in the bilayer membrane. These experiments revealed interaction of alamethicin only with the phospholipid head groups, at least in the absence of a transmembrane potential; no perturbation of the lipid acyl chains was noted. These findings reinforce earlier conclusions (Lau & Chan, 1975, 1976) that alamethicin is principally a surface-active peptide and its partitioning into the hydrocarbon region of the bilayer is minimal.

Materials and Methods

The alamethicin used in this study was a generous gift of Dr. G. B. Whitfield, Jr., and Dr. J. E. Grady of the Upjohn Co. and was used without further purification. Dimyristoylphosphatidylcholine (DML) was obtained from Sigma Chemicals and was checked for purity by thin-layer chromatography. Bis(perdeuteriomyristoyl)phosphatidylcholine (DML-d_{54}) was purchased from Serdary Chemicals, Canada. D_2O used was from Aldrich Chemicals.

Multilamellar dispersions used in this study were made as follows: first, the lipid, or the lipid–alamethicin mixture, was dissolved in methanol in an NMR tube. The solvent was then evaporated off with dry nitrogen, and the tube was kept under vacuum for at least 8 h. The thin film thus formed was hydrated with a 25 mM Tris buffer solution (pD 7.4) in D_2O by repeated vortexing on a mixer and gentle warming with a heat gun. The vortexing was continued for about 5 min until a uniform white suspension was obtained. The samples were always fully hydrated and were typically 1:10 w/v in lipid to water. Proper hydration and prolonged vortexing were important in obtaining a uniform lipid mixture. When hydration or mixing was incomplete, nonaxial or isotropic features were noted in the ^{31}P NMR spectra. Two alamethicin concentrations were used in the current work, corresponding to lipid to peptide molar ratios of 15:1 and 2:1. Lipid-gramicidin S and lipid–chlorophyll *a* mixtures were also prepared by using the same procedure. Samples for ^{2}H NMR studies were prepared in exactly the same manner except DML-d_{54} was used instead of the regular lipid and deuterium-depleted water was substituted for D_2O. In these studies, the samples were also checked for the integrity and nature of the bilayer by examining their ^{31}P spectra. For measurements at higher ionic strengths, the buffer solution was made in 0.5 M NaCl. All samples were maintained at 30 °C until NMR experiments were carried out. For variable-temperature experiments, the rate of heating was controlled at 1 °C/min, and the samples were equilibrated for at least 10 min prior to making measurements at any given temperature.

^{1}H and ^{2}H NMR spectra were acquired at 11.74 T (500.13-MHz ^{1}H frequency and 76.78-MHz ^{2}H frequency) on a Bruker WM 500 spectrometer equipped with an Aspect 2000 data system and a variable-temperature controller that is capable of maintaining the temperature of the sample constant within 0.5 °C. The usual parameters were used to obtain the ^{1}H spectra. ^{2}H spectra were acquired with a high-power home-built probe (Müller & Chan, 1983) using the standard quadrupolar echo sequence (Davis et al., 1976). The spectral width was 166 kHz, and a refocusing time of 50 μs was used. ^{31}P spectra were acquired either at 11.74 T (202.49-MHz ^{31}P frequency) or at 4.7 T (80.99-MHz ^{31}P frequency). A spectral width of 50 kHz, a 75° pulse, a relaxation delay of 1.5 s, and gated broad-band decoupling of 10 W were employed to obtain the spectra at 11.74 T on the Bruker WM 500. The ^{31}P experiments at 4.7 T were carried out on a home-built wide-bore 200-MHz spectrometer for solids. Signals were recorded by using a high-power ^{31}P observe pulse (90° pulse, 5 μs) with proton decoupling (90° pulse, 4 μs).

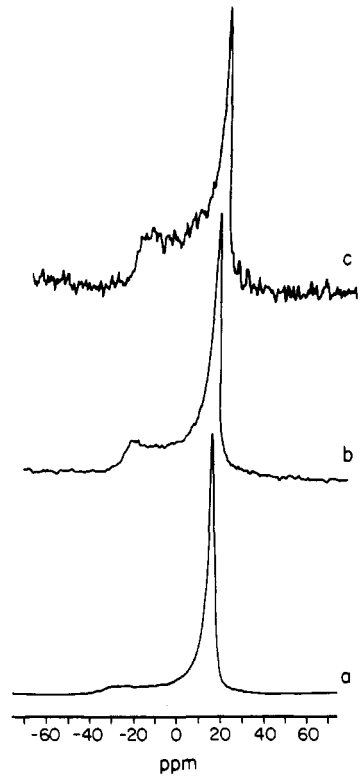

FIGURE 1: ^{31}P NMR spectra of lipid head-group phosphorus in fully hydrated DML–alamethicin mixtures. The spectra were recorded at 202.49-MHz ^{31}P frequency at 26 °C. (a) DML; (b) 15:1 DML:alamethicin mole ratio; (c) 2:1 DML:alamethicin mole ratio.

For simulation of the ^{31}P spectra, a computer program coded by Jack Freed and co-workers at Cornell University (Campbell et al., 1979) for the calculations of ESR line shapes on a PDP 1134 was adapted for ^{31}P NMR simulation on a VAX 11/780 computer to suit our current needs.

Raman spectra of the lecithin dispersions were recorded on a Spex Industries Model 14018 double monochromator equipped with 2400 line/mm holographic gratings and a Hammamatsu R955 photomultiplier tube. Spectral slit widths were 4 cm^{-1}. A Spectra-Physics Model 170 argon ion laser was used to pump rhodamine 590 in a Spectra-Physics Model 375a dye laser. The excitation wavelength in the Raman experiments was 514.5 nm; laser power was maintained at approximately 300 mW. A Spex Industries Model SC-32 SCAMP spectrometer controller/data processor was used for repetitive spectral scanning and for data manipulation. Spectra were accumulated in intervals of 1 cm^{-1} at a scanning rate of 1 cm^{-1}/s.

Results

^{31}P NMR Studies. Figure 1 shows a typical spectrum of fully hydrated DML multilayers above the gel–liquid-crystalline phase transition temperature (T_c) in the absence and

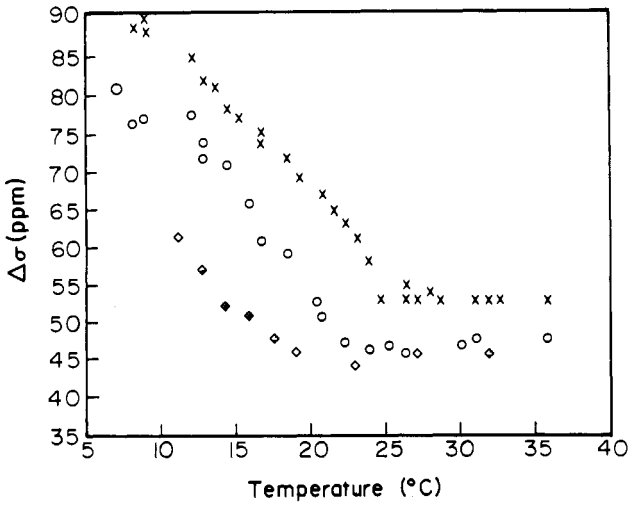

FIGURE 2: Plot of $\Delta\sigma$ vs. temperature illustrating the effect of alamethicin on the ^{31}P chemical shift anisotropy of the lipid phosphorus in DML multilayers. (×) No alamethicin added; (○) 15:1 DML:alamethicin mole ratio; (◇) 2:1 DML:alamethicin mole ratio.

presence of alamethicin. The ^{31}P spectra of head groups in phospholipid multilayers exhibit an axially symmetric powder pattern (Seelig, 1978). Since the static chemical shift tensor of the phosphate moiety is nonaxial, there must exist fast motions which allow the chemical shift tensor to be averaged. The major motions that contribute to this averaging are the rapid intramolecular isomerization of the head group and the rotational diffusion of the lipid molecules about the director. In such a case, only the parallel and perpendicular components of the motionally averaged σ tensor can be measured; these are related to the static chemical shift tensor components (σ_{ii}, $i = x, y,$ or z) as follows:

$$\sigma_{\parallel} = \overline{\sin^2\theta\cos^2\phi}\sigma_{xx} + \overline{\sin^2\theta\sin^2\phi}\sigma_{yy} + \overline{\cos^2\theta}\sigma_{zz} \quad (1)$$

$$\sigma_{\perp} = (1/2)[(\sigma_{xx} + \sigma_{yy} + \sigma_{zz}) - \sigma_{\parallel}]$$

The bar denotes time averages and θ and ϕ are tilt angles for the head group. The anisotropy in the motionally averaged tensor is the chemical shift anisotropy $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$. From the spectra shown in Figure 1, $\Delta\sigma$ can be determined from the splitting between the edges of the spectrum at the half-height of the low-frequency "foot".

The dependence of the experimentally measured $\Delta\sigma$ on the molecular motions offers a way to compare the motional states of the phospholipid head groups in the absence and presence of the peptide. From Figure 1, we ascertain that there is no qualitative change in the line shape of the spectrum even at a very high concentration of alamethicin (2 mol of lipid/mol of peptide). This important observation indicates that the integrity of the bilayer phase is maintained in the presence of even high concentrations of alamethicin. Throughout the work, we have obtained no evidence for lipid polymorphism as a result of interaction of alamethicin with DML multilayers. However, the comparison of spectra with and without alamethicin reveals that $\Delta\sigma$ decreases significantly when alamethicin is present. Above T_c, this effect appears to be already saturated at the lipid to alamethicin ratio of 15:1.

Figure 2 summarizes the temperature dependence of $\Delta\sigma$ for DML multilayers in the presence and absence of alamethicin. The sharp rise in $\Delta\sigma$ at 24 °C corresponds to the gel–liquid-crystalline phase transition of the phospholipids and reflects the change in the motional state of the head groups. Our results show that the onset of this thermal phase transition is not appreciably affected by alamethicin even at high concentrations of the peptide.

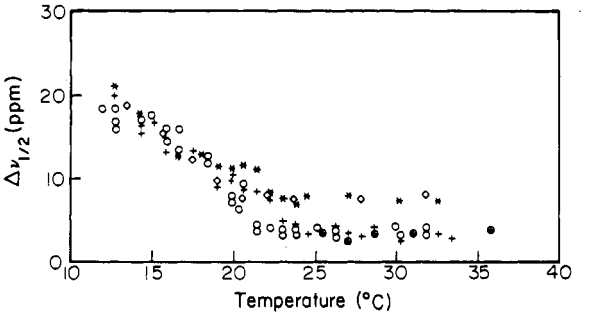

FIGURE 3: Plot of $\Delta\nu_{1/2}$ vs. temperature for alamethicin containing DML multilayers. (+) No alamethicin, with ^1H decoupling; (asterisks) no alamethicin, no decoupling; (○) 15:1 lipid:alamethicin mole ratio, with decoupling; (◇) 15:1 lipid:alamethicin mole ratio, no decoupling.

Another parameter that is often used for characterization of ^{31}P spectra of multilamellar dispersions is the width at half-height, $\Delta\nu_{1/2}$ (Seelig & Gally, 1976). This is an empirical parameter; it is not a measure of true line widths of various contributions to the superimposed powder pattern. It is, however, dependent on the residual ^1H–^{31}P dipolar coupling which, in turn, depends on the extent of motional averaging in the system. In principle, the magnitude of this residual coupling may be estimated from the spectra obtained with and without ^1H decoupling. Figure 3 shows the temperature dependence of $\Delta\nu_{1/2}$ of DML multilayers, in both the presence and absence of 10-W broad-band proton decoupling. As expected, the decoupling of ^{31}P from protons above T_c results in lowering of $\Delta\nu_{1/2}$. However, with the decoupling power used in these experiment, it was not possible to eliminate the strong dipolar ^1H–^{31}P couplings in the gel phase. In any case, we noted no change in $\Delta\nu_{1/2}$ with the addition of alamethicin (Figure 3). This indicates that the nature of the ^1H–^{31}P coupling and the molecular motions of the lipid chains are not significantly affected by the peptide.

Since most conductance measurements on alamethicin have been undertaken in the presence of salts, our measurements were also performed in 0.5 M NaCl solution. At a lipid to alamethicin ratio of 15:1, we observed no change in $\Delta\sigma$ over the entire range of temperatures investigated.

Simulation of the ^{31}P NMR Spectra. We have simulated the ^{31}P powder pattern using the approach suggested by Freed and co-workers (Campbell et al., 1979). These workers extended the earlier formalism of Mason, Polnaszek, and Freed (Mason et al., 1974) for the simulation of electron spin resonance (ESR) spectra of slowly tumbling nitroxide spin probes undergoing highly anisotropic rotational reorientation to the calculation of $I = {}^1/_2$ NMR line shapes of a molecular system wherein a group to which the nuclear spin probe is attached is undergoing relatively rapid rotation about an internal axis (a bond) which is tilted at an arbitrary angle from the principal axes of the overall rotational diffusion of the molecule. The complex set of motions which average the chemical shielding tensor is described by three diffusion constants. These are the very rapid diffusion constant for the bond reorientation(s) (D_I), the rotational diffusion constant for the fast overall rotational motion of the molecule ($D_{\parallel}$), and the slow molecular tumbling diffusion constant ($D_{\perp}$). In the present instance, the principal axes of the ^{31}P chemical shift tensor are not coincident with the molecule fixed-diffusion axes; two angles (θ and ϕ) which relate the two sets of axes have been defined by Freed et al. (Figure 4) and are termed tilt angles. In the algorithm written by Freed and co-workers, the spin Hamiltonian for the problem is set up in a proper coordinate system to account for the tilted diffusion frame of the molecule. Local motions are incorpo-

Table I: Calculated $\Delta\sigma$ Values for the Various ^{31}P NMR Spectra Simulated in Figure 6

figure	spectrum	T_2^{-1} (G)	$D_\parallel$ (s^{-1})	$D_\perp$ (s^{-1})	D_I (s^{-1})	θ (deg)	ϕ (deg)	$\Delta\sigma$ (ppm)	inferences
6A	j	0.1	1×10^7	1.0	1×10^{10}	83	75	54	$\Delta\sigma$ is highly sensitive to angles θ and ϕ; $\Delta\sigma$ decreases when θ decreases and/or ϕ increases
	i	0.1	1×10^7	1.0	1×10^{10}	83	83	49	
	h	0.1	1×10^7	1.0	1×10^{10}	83	73	56.5	
	g	0.1	1×10^7	1.0	1×10^{10}	83	63	69.5	
	f	0.1	1×10^7	1.0	1×10^{10}	83	53	86.5	
	e	0.1	1×10^7	1.0	1×10^{10}	90	75	58.5	
	d	0.1	1×10^7	1.0	1×10^{10}	85	75	56.5	
	c	0.1	1×10^7	1.0	1×10^{10}	75	75	40.5	
	a	0.1	1×10^7	1.0	1×10^{10}	65	75	(0)	
	b	0.1	1×10^7	1.0	1×10^{10}	55	75	(−34)	
		0.1	1×10^5	1.0	1×10^{10}	83	75	54.5	for high D_I, no effect on $\Delta\sigma$ is seen with change in $D_\parallel$
		0.1	1×10^3	1.0	1×10^{10}	83	75	54	
		0.1	10	1.0	1×10^{10}	83	75	54	
		0.1	0.1	1.0	1×10^{10}	83	75	54.5	
6B	a	0.1	1×10^4	1.0	1×10^{10}	83	75	54	$\Delta\sigma$ is sensitive to $D_\parallel$ only when the latter is changed by ~4 orders of magnitude
	b	0.1	1×10^4	1.0	1×10^8	83	75	54.5	
	c	0.1	1×10^4	1.0	1×10^6	83	75	55	
	d	0.1	1×10^4	1.0	1×10^4	83	75	undefined	
	e	0.1	1×10^4	1.0	1×10^2	83	75	undefined	
	f	0.1	1×10^4	1.0	8×10^5	83	75	56	
	g	0.1	1×10^4	1.0	4×10^5	83	75	56.5	
	h	0.1	1×10^4	1.0	1×10^5	83	75	59.5	
	i	0.1	1×10^4	1.0	6×10^4	83	75	66.5	
	j	0.1	1×10^4	1.0	2×10^4	83	75	undefined	
6C	a	0.01	1×10^7	1.0	1×10^{10}	83	75	50	T_2^{-1} affects "foot height"; only a small effect on $\Delta\sigma$ is seen
	b	0.02	1×10^7	1.0	1×10^{10}	83	75	50.5	
	c	0.03	1×10^7	1.0	1×10^{10}	83	75	51	
	d	0.05	1×10^7	1.0	1×10^{10}	83	75	52	
	e	0.15	1×10^7	1.0	1×10^{10}	83	75	56	
	f	0.2	1×10^7	1.0	1×10^{10}	83	75	57	
	g	0.3	1×10^7	1.0	1×10^{10}	83	75	61	
	h	0.5	1×10^7	1.0	1×10^{10}	83	75	undefined	
	i	1.0	1×10^7	1.0	1×10^{10}	83	75	undefined	

rated by solving a stochastic Liouville's equation to obtain coefficients of the eigenfunction expansion of the density matrix. The absorption spectrum is then calculated by using these coefficients. Spectral simulations are obtained with $D_\parallel$, $D_\perp$, D_I, θ, ϕ, and T_2^{-1} as input parameters where T_2^{-1} is related to the line width of the constituent Lorentzian envelopes that make up the powder pattern. For simplicity, we have ignored the possibility of the microscopic ordering potential at the position of the phosphate moiety. Campbell et al. (1979) have provided justification for this assumption, despite the experimental evidence for microscopic ordering of fully hydrated lecithin bilayers. Thus, our simulations are based on the model of anisotropic but unrestricted diffusion of a cylinder.

Figure 5 compares a calculated ^{31}P spectrum with an experimental spectrum of a sample with a 15:1 DML:alamethicin molar ratio acquired on a 4.7-T solid-state NMR spectrometer. The experimental spectrum in this figure has a superior line shape to the ones presented earlier since it was acquired with high-power amplifiers on both the observe and the decoupler channels. The 90° pulse for the ^{31}P nucleus on this instrument is 5 μs, and for ^1H, it is 4 μs. The σ value is not significantly affected by this change in line shape. The experimental spectrum is closely fitted by the simulated spectrum calculated with $D_I = 1 \times 10^{10}$ s^{-1}, $D_\parallel = 1 \times 10^7$ s^{-1}, $D_\perp = 1$ s^{-1}, $T_2^{-1} = 0.05$ G, $\theta = 79°$, and $\phi = 78°$.

The parametric dependence of $\Delta\sigma$ is summarized in Table I, and the corresponding spectra are presented in Figure 6. The most important observation from these spectral simulations is that the value of $\Delta\sigma$ is significantly affected by very small ($\leq 5°$) changes in the tilt angles. This is true even when $\Delta\sigma$ is small, corresponding to the experimentally observed values at high temperatures (above T_c). Under these conditions, the conformation of the head groups is flexible. The

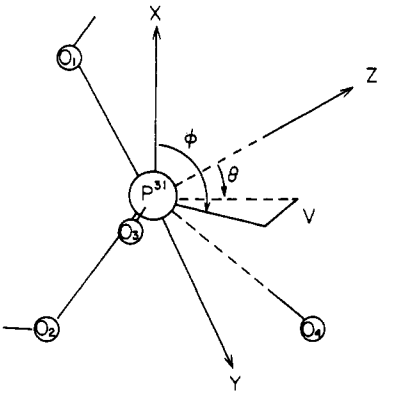

FIGURE 4: Schematic representation of the PO_4 group in the DML molecule. x, y, and z denote the principal axes of the chemical shift tensor of the ^{31}P nucleus, and θ and ϕ define the orientation of the diffusion axis v. Axis y lies within the O–P–O plane and approximately bisects the O–P–O angle, where the O's are the nonesterified oxygens (Campbell et al., 1979).

sensitivity of $\Delta\sigma$ to this conformation indicates that the reduction in $\Delta\sigma$ on adding alamethicin could easily be caused by a change in the average tilt angles of the lipid molecules in the bilayer. In fact, the spectrum of the alamethicin–DML system can be simulated with $\theta = 79°$ and $\phi = 78°$, a small departure from the values of $\theta = 83°$ and $\phi = 75°$ needed to reproduce the anisotropy for pure DML dispersions. Table I also shows that the value of $\Delta\sigma$ is insensitive to the diffusion constants for all $D_I \geq 1 \times 10^6$ s^{-1} and $D_\parallel \geq 1 \times 10^4$ s^{-1}. Since the normal values of D_I and $D_\parallel$ are $\sim 10^{10}$ and $\sim 10^8$ sec^{-1}, respectively (Campbell et al., 1979), it is apparent that a change in $\Delta\sigma$ can only be brought about by 3–4 orders of magnitude reduction in the values of these diffusion constants.

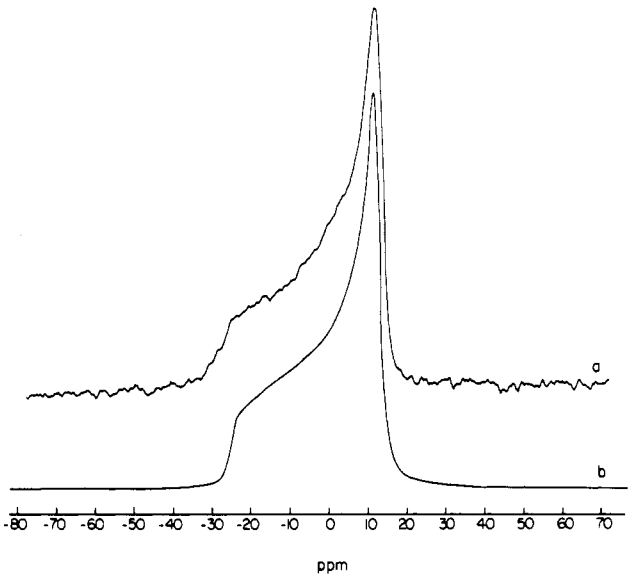

FIGURE 5: (a) High-power (~200-W) proton-decoupled ^{31}P NMR spectrum at 80.97 MHz. Temperature, 25 °C. (b) Theoretical spectrum obtained from a density matrix calculation to simulate the ^{31}P line shape of the spectrum shown in (a). Input parameters are $\theta = 79°$, $\phi = 78°$, $D_{\parallel} = 1 \times 10^7$ s^{-1}, $D_{\perp} = 1$ s^{-1}, $D_I = 1 \times 10^{10}$ s^{-1}, and $T_2^{-1} = 0.05$ G.

However, the calculated spectra of Figure 6 show that any large reductions in the values of diffusion constants will lead to gross changes in the line shape.

The effect of T_2^{-1} on $\Delta\sigma$ is minor; a change of only 4 ppm is seen, for example, when T_2^{-1} is changed from 0.01 G to 0.1 G. A change in T_2^{-1} can cause large variations in line shapes, however; the low-field foot of the spectrum is quite prominently affected. Although the formalism used in the present work assumes orientation-independent T_2^{-1} values, an assumption which is not rigorously correct, the angular dependence of T_2^{-1} has been shown to have only minor effects at the edges of the ^{31}P powder spectrum.

Comparison with Gramicidin S and Chlorophyll a. For comparison and contrast with the ^{31}P NMR results obtained for alamethicin, we have conducted related experiments with amphiphiles that are known to interact with phospholipid multilayers. Experiments with gramicidin S reveal lipid polymorphism even at a 15:1 lipid to peptide ratio. At higher concentrations, the appearance of a large isotropic peak indicates extensive micellization (R. Zidovetzki et al., unpublished results). Similar experiments with chlorophyll *a* show that the bilayer structure is preserved in lipid–chlorophyll mixtures but a large shift in the phase transition temperature is seen (P. Dea et al., unpublished results). These results offer an important contrast to those obtained for DML–alamethicin mixtures.

^2H NMR Studies. Figure 7 compares the ^2H NMR spectrum of DML-d_{54} above the phase transition temperature in the absence and presence of alamethicin. Each of these spectra

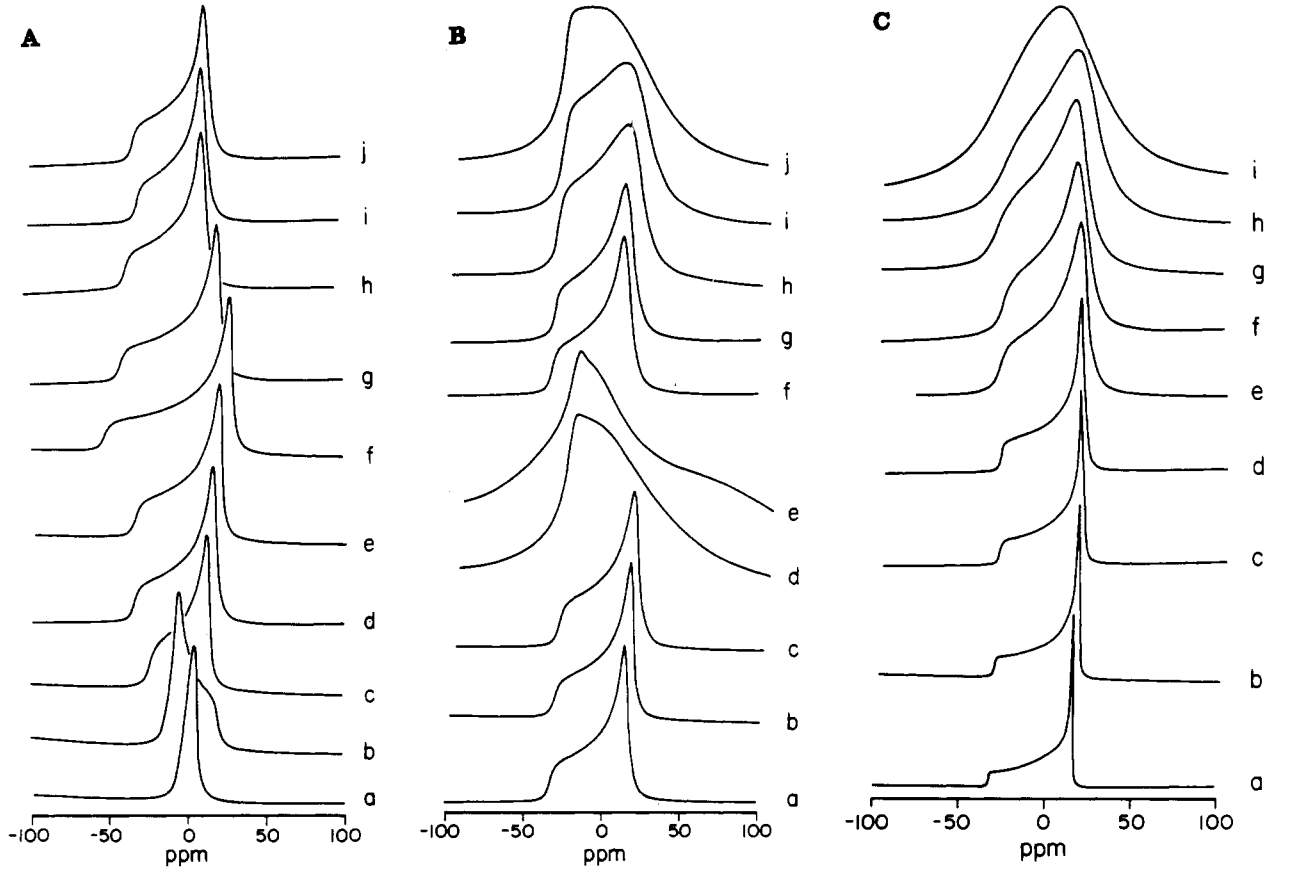

FIGURE 6: (A) Simulated spectra illustrating the sensitivity of $\Delta\sigma$ and the ^{31}P line shape to the values of θ and ϕ for $D_{\parallel} = 1 \times 10^7$ s^{-1}, $D_{\perp} = 1$ s^{-1}, $D_I = 1 \times 10^{10}$ s^{-1}, and $T_2^{-1} = 0.1$ G. (a) $\theta = 65°$, $\phi = 75°$; (b) $\theta = 55°$, $\phi = 75°$; (c) $\theta = 75°$, $\phi = 75°$; (d) $\theta = 85°$, $\phi = 75°$; (e) $\theta = 90°$, $\phi = 75°$; (f) $\theta = 83°$, $\phi = 53°$; (g) $\theta = 83°$, $\phi = 63°$; (h) $\theta = 83°$, $\phi = 73°$; (i) $\theta = 83°$, $\phi = 83°$; (j) $\theta = 83°$, $\phi = 75°$. (B) Theoretical spectra calculated with different values of D_I. For all spectra, $D_{\perp} = 1$ s^{-1}, $D_{\parallel} = 1 \times 10^4$ s^{-1}, $\theta = 83°$, $\phi = 75°$, and $T_2^{-1} = 0.1$ G. (a) $D_I = 1 \times 10^{10}$ s^{-1}; (b) $D_I = 1 \times 10^8$ s^{-1}; (c) $D_I = 1 \times 10^6$ s^{-1}; (d) $D_I = 1 \times 10^4$ s^{-1}; (e) $D_I = 1 \times 10^2$ s^{-1}; (f) $D_I = 8 \times 10^5$ s^{-1}; (g) $D_I = 4 \times 10^5$ s^{-1}; (h) $D_I = 1 \times 10^5$ s^{-1}; (i) $D_I = 6 \times 10^4$ s^{-1}; (j) $D_I = 2 \times 10^4$ s^{-1}. (C) Simulated spectra illustrating the sensitivty of the ^{31}P line shape to the spin–spin relaxation time of the phosphorus for $D_{\parallel} = 1 \times 10^7$ s^{-1}, $D_{\perp} = 1$ s^{-1}, $D_I = 1 \times 10^{10}$ s^{-1}, $\theta = 83°$, and $\phi = 75°$ [see Figure 6B(a) for $T_2^{-1} = 0.1$ G]. (a) $T_2^{-1} = 0.01$ G; (b) $T_2^{-1} = 0.02$ G; (c) $T_2^{-1} = 0.03$ G; (d) $T_2^{-1} = 0.05$ G; (e) $T_2^{-1} = 0.15$ G; (f) $T_2^{-1} = 0.2$ G; (g) $T_2^{-1} = 0.3$ G; (h) $T_2^{-1} = 0.5$ G; (i) $T_2^{-1} = 1.0$ G.

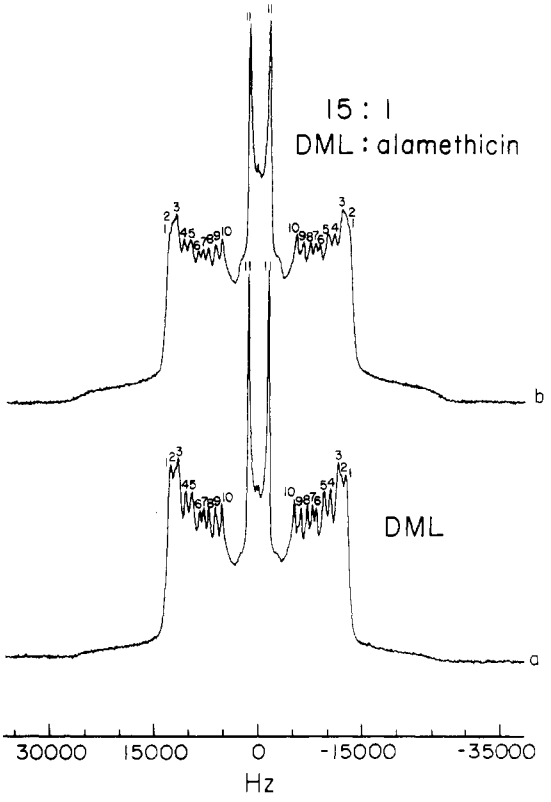

FIGURE 7: ^{2}H NMR spectra of DML multilayers with perdeuterated hydrocarbon chains. (a) DML-d_{54} only. (b) 15:1 DML-d_{54}:alamethicin mole ratio. Tentative assignments of peaks 1 through 11 are given in Table II.

represents the superposition of axially averaged powder patterns arising from the different deuterons for each CD_2 segment along the chains. The CD bond order parameter for the ith segment, S_{CD}^i, can be derived from the quadrupolar splitting observed at the perpendicular orientation, where

$$\Delta\nu_D^i = (3/4)(e^2qQ/h)S_{CD}^i$$

and e^2qQ/h is the CD deuteron quadrupolar coupling constant. It has been shown by specific deuteration of lipids that the order parameter profile along the chains of a phospholipid molecule in a multilayer shows a plateau for the methylene segments near the glycerol backbone (Seelig, 1977). The corresponding quadrupolar patterns due to these segments are nearly coincident with one another, and the signals at the perpendicular orientations overlap near the edge of the spectrum. Since the lower parts of the chains are more disordered, the quadrupolar patterns from these methylene segments show well-resolved peaks at the perpendicular orientation. The observed quadrupolar splittings for DML-d_{54} and a tentative assignment based on the specific deuteration experiments reported earlier for DPL (Seelig & Seelig, 1974) are given in Table II.

A comparison of spectra a and b of Figure 7 shows that alamethicin has only a minimal effect on the hydrocarbon chains of the bilayer. The only qualitative change is seen for the outermost peak which arises from the methylene segments near the glycerol backbone. The remaining peaks all remain unchanged in the presence of alamethicin. Table II summarizes the $\Delta\nu_D^i$ values for the two samples; they are identical within the limits of experimental error. More importantly, perhaps, the ^{2}H spectrum for the sample containing alamethicin does not show the typical broadening observed when proteins are incorporated into bilayers (Seelig & Seelig, 1978; Rice et al., 1979).

Table II: Detailed Comparison of the ^{2}H Quadrupolar Splittings Observed for DML-d_{54} Multilamellar Dispersions with and without Alamethicin

peak	tentative assignment of chain deuterons to C no.[a]	$\Delta\nu_D^i$ (kHz) without alamethicin	$\Delta\nu_D^i$ (kHz) with alamethicin[b]	% difference
1	2a, 3–5	25.3	25.9	2.4
2	7b, 6a, 6b	24.0	25.2	5.0
3	8b, 7a, $2b_1$	23.0	23.9	3.9
4	9b, 8a	20.8	21.8	4.8
5	10b, 9a	19.0	19.8	4.2
6	10a	16.7	17.5	4.8
7	11b	15.6	16.3	4.5
8	11a	14.2	14.6	2.8
9	12b, b_2	12.3	12.7	3.2
10	12a	10.5	10.8	2.8
11	13	2.9	2.9	(1.4)

[a] a and b denote the two chains. [b] 15:1 lipid:alamethicin mole ratio.

Raman Studies. We have also investigated the effect of alamethicin on the motional states of the hydrocarbon chains by Raman spectroscopy. The Raman intensity ratios I_{1065}:I_{1087}, I_{2890}:I_{2850}, and I_{2850}:I_{2930} have been shown by Lis et al. (1976) to be sensitive to the conformation of the phospholipid chains, particularly to the gauche:trans ratio. Our studies show that these ratios are unchanged upon addition of alamethicin to lipid bilayers. We find that I_{1065}:I_{1087}, I_{2890}:I_{2850}, and I_{2850}:I_{2930} are 1.07, 0.95, and 1.5, respectively, for pure DML at 25 °C; the corresponding values are 1.0, 1.0, and 1.35, respectively, for a 15:1 DML–alamethicin mixture at the same temperature. These observations are consistent with the ^{2}H NMR results and the conclusion that the molecular packing of the hydrocarbon chains is not perturbed by alamethicin.

Discussion

The present work was initiated in order to resolve the question of the disposition of the alamethicin molecule relative to the bilayer in lipid dispersions containing the peptide. Toward this end, we have undertaken direct spectroscopic investigations of the fully hydrated alamethicin–lipid system at different conditions of temperature and peptide concentration.

Even at a lipid to alamethicin ratio of 2:1, the characteristic peaks attributed to the peptide were not evident in the 500-MHz proton spectrum of the mixture. Only a broad spectrum due to the lipids in the multilayers is observed (not shown). This conspicuous lack of a proton spectrum for the peptide constitutes strong evidence that alamethicin is highly aggregated and motionally hindered in the dispersion.

The interaction of alamethicin with lipid head groups was established by ^{31}P NMR measurements. Results of these experiments indicate that the bilayer phase of DML is maintained at all temperatures, even at the highest alamethicin to lipid ratio investigated (1:2) and in the absence or presence of salt. There is no evidence for lipid polymorphism such as has been obtained for gramicidin S–lipid (R. Zidovetzki et al., unpublished results), and gramicidin A–lipid (Van Echteld et al., 1981) systems.

While our experiments reveal no major perturbation of the DML bilayer structure by alamethicin, the observed change in the ^{31}P $\Delta\sigma$ indicates that the peptide does interact with the bilayer at the lipid–water interface. Spectral simulations indicate that the lowering of $\Delta\sigma$ can be accounted for by slight changes in the orientation of the headgroup vis ã vis the bilayer plane in the presence of the peptide.

The gel–liquid-crystalline phase transition temperature is not appreciably modified by the addition of alamethicin to

DML multilayers as has been observed, for example, in the case of chlorophyll *a* containing bilayers. We noted no measurable change in T_c at an alamethicin to lipid ratio of 1:15; at the higher ratio (1:2), a shift of about 4 °C is seen. Such minor effects of T_c are expected when surface-active peptides bind at the aqueous interface. Larger perturbations on the phase transition temperature are observed for molecules such as chlorophyll *a* which intercalate into the bilayer even at lower mole ratios.

Conclusive evidence for the nonperturbation of the lipid chains by alamethicin comes from ^2H NMR of the chain-perdeuterated lipid. These experiments revealed broadening of the peaks only at the extreme edges of the spectrum, again evidence for support of an interaction near the bilayer interface. Interaction of alamethicin with the lipid bilayer does not affect the relaxation times of the side-chain deuterons either. Incorporation of peptides to an appreciable extent into the bilayer would have produced significant broadening of the various resonances even when the order parameters are not affected, as has been observed for lipid–protein recombinants (Seelig & Seelig, 1978; Rice et al., 1979).

Conclusions from the above ^2H NMR results have been further complemented by Raman studies of the phospholipid chains. The Raman intensity ratios I_{1065}:I_{1087}, I_{2890}:I_{2850}, and I_{2850}:I_{2930} have been shown by Lis et al. (1976) to be sensitive to gauche:trans ratios and the conformation of the phospholipid chains. In agreement with the NMR results, the Raman studies in the presence of alamethicin show no change in the above ratios, indicating that the presence of alamethicin does not change the conformation of the lipid side chains.

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